Application No. 09/825,713
Reply to Office action of June 3, 2003

Group Art Unit: 1636
Examiner: Konstantina T. Katcheves
Atty. Docket No. 104036-14

REMARKS

Claims 1-4, 8-13, 16 and 17 are pending in the application. The oath or declaration has been deemed defective under 37 CFR 1.67(a). Claims 1-4, 8-13, 16 and 17 stand rejected under 35 U.S.C. § 112, first paragraph, because the invention does not reasonably provide enablement for methods drawn to the treatment of human subjects. Claims 1-4 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Eglitis *et al.* (PNAS Vol. 94 1997).

By this response, Applicants hereby amend claim 1, cancel claims 9 and 17, and present new claims 20-24 to better define the present invention. Support for the amendment to claim 1 can be found throughout the specification and in the claims as originally filed. For example, support for the phrase "selecting mammalian stem cells of myeloid origin capable of differentiating into neuronal cells" can be found at page 12, line 6 through page 14, line 2. New claims 20-24 are directed to specific markers that can be used to select for mammalian stem cells of myeloid origin capable of differentiating into neuronal cells. Support for these claims can be found on page 12, lines 12-24, page 12, lines 27-32, page 13, lines 16-21 of the specification. Accordingly, no new matter has been added by these amendments. Applicants respectfully request entry of new claims 20-24 into the file of record.

While Applicants believe that the originally presented claims are patentable over all of the art of record and otherwise in view of all references submitted by Applicants, the claims have nonetheless been amended as follows in order to expedite the application toward allowance. The amendments are therefore made without prejudice or disclaimer, and Applicants reserve the right to pursue the original scope of the claims as provided prior to the amendments, such as through continuation practice.

Applicants respectfully traverse the Examiner's rejections and request reconsideration of the application in view of the amendments made above and the remarks that follow.

Oath/Declaration

The Examiner states that the oath or declaration is defective and that a new oath or declaration in compliance with 37 CFR 1.67(a) identifying this application by application number and filing date is required. In particular the Examiner points out asserts that:

> The oath or declaration is defective because: It does not identify the mailing or post office address of each inventor….The mailing or post office address may be provided in an application data sheet or a supplemental oath or declaration.

While the Applicants contends that the oath submitted April 4, 2001 along with the filing of the present application is fully compliant with 37 CFR 1.67(a) and includes the mailing address for each inventor including the ZIP Code designations, an application data sheet is nonetheless submitted with this response as requested by the Examiner.

Rejections under 35 U.S.C. § 112, 1st paragraph

Claims 1-4, 8-13, 16 and 17 stand rejected under 35 U.S.C. § 112, first paragraph. In particular, the Office Action states that "the specification, while enabling for methods drawn to mice and rats, does not reasonably provide enablement for methods drawn to the treatment of human subjects." The Office Action further refers to MPEP 2164.01 citing that:

> A conclusion of lack of enablement means that, based on the evidence regarding each of the above factors, the specification, <u>at the time the application was filed,</u> would not have taught one skilled in the art how to make and/or use the full scope of the claimed invention without undue experimentation. *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993).

The Examiner further states:

> Applicant should note that the Mezey et al. reference relied upon as evidence of enablement of the invention for mammals other than mice and rats was published three years after the priority date of the present application. Thus, the disclosure of Mezey et al. is inappropriate evidence of the state of the art.

Applicants respectfully traverse this rejection and address the concerns of the Examiner. The Mezey *et al.* article was not cited to show the state of the art at the time the Applicants' application was filed, but rather that the methods disclosed in the Applicants' application yielded similar results in humans as the Applicants had shown in

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rats and mice. This article also shows that the human data was obtained without undue experimentation. The human experiments shown in the February 2003 Mezey *et al.* reference, which was submitted to PNAS in October 2002, follow directly from the animal experiments disclosed in the Mezey December 2000 Science paper. Thus, the Mezey *et al.* 2003 reference shows that in less than two years, a lab using methods similar to those disclosed in the Applicants' application could obtain similar results in humans. A time span of two years to go from animal to human data is reasonable for inventions that are well conceived. Thus, utilizing the methods in humans is a straightforward application of the technology.

Furthermore, the Mezey *et al.* experiments demonstrate that well characterized animal models can be used to successfully predict human results without undue experimentation. It is well established that enablement is not precluded by the need for experimentation, even a large quantity of experimentation, if the specification, in combination with the knowledge available in the art, provides guidance regarding how to carry out the experimentation such that the experimentation is not "undue." Moreover, a "considerable amount of experimentation is permissible, if it is merely routine" *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (citing *In re Angstadt*, 537 F.2d 489, 502-504, 190 USPQ 214, 218 (CCPA 1976)).

In addition, the first paragraph of 35 U.S.C. §112 requires nothing more than objective enablement. How such a teaching is set forth, by specific example or broad terminology, is not important. *In re Marzocchi*, 169 USPQ 367 (CCPA 1971). Compliance with the first paragraph does not turn on whether a specific example or working example is disclosed. *In re Borkowski*, 164 USPQ 642 (CCPA 1970).

Thus, the specification of the instant invention provides adequate teaching and guidance to enable one of ordinary skill in the art to make and use the claimed methods of the instant invention. Applicants reiterate that the relative level of skill in the art is fairly sophisticated, and consequently, a person having ordinary skill in this art would be

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familiar with the techniques necessary to utilize this invention in humans. Accordingly, it would not constitute undue experimentation for a skilled artisan to deliver a therapeutically effective amount of mammalian stem cells into the nervous systems of other mammals, in addition to mice and rats, by following the teachings of the Applicant's specification.

The specification is replete with teachings on how to isolate progenitor and stem cells using markers, such as CD34 and KDR, which are found on human lineage committed progenitor cells (*See*, for example, page 12, lines 10-24 of the specification). The specification teaches multiple ways of selecting for human progenitor cells, such as using immunofluorescently labeled monoclonal antibodies or a two-step purification of low density human bone marrow cells by negative immunomagnetic selection and positive dual-color florescence activated cell sorting. (*See*, for example, page 13, lines 7-28 of the specification). The specification also discloses methods of culturing the stem cells (*See*, page 14, lines 4-33) and methods of transplanting the cultured stem cells into a subject (*See*, page 15, line 26 through page 16, line 2).

For all of these foregoing reasons, Applicant respectfully requests that the Examiner withdraw all rejections under 35 U.S.C. § 112, first paragraph.

Rejections under 35 U.S.C. § 102(b)

Claims 1-4 have been rejected under 35 U.S.C. § 102(b) as being anticipated by Eglitis *et al.* (PNAS Vol. 94 1997). In particular, the Office Action states that:

> Eglitis et al. discloses the transplantation of myeloid cells marked with a retroviral vector into the brains of mice. These cells are capable of migration to discrete parts of the brain and express the exogenous gene. Eglitis further discloses that myeloid derived cells acquire microglial antigenic markers and finds hematopoietically derived microglia in the brains of rats. See pages 4080-4081 and page 4082, column 1. (Emphasis added.)

Applicants respectfully traverse this rejection, as Eglitis *et al.* fails to teach each and every claim element of the claimed invention. The amendments submitted herewith

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further clarify the differences between the Eglitis reference and the present invention. To expedite prosecution, Applicants have amended claim 1 to recite the step of *"selecting mammalian stem cells* of myeloid origin capable of differentiating into neuronal cells."

Eglitis teaches methods of *tagging*, either with a retroviral vector or by transplant of male cells into a female recipient, hematopoietic cells. In the first tagging method, cytoplasmic tagging, Eglitis discloses tagging bone marrow cells upon harvest with retroviral vector *without any prior selection* of the bone marrow cells. Eglitis teaches collecting the *unselected bone marrow cells*, which had been tagged with retroviral vector, by gentle aspiration. In the second tagging method, nuclear tagging, bone marrow cells from male mice are harvested *without any selection* and injected into female mice. The tagging techniques disclosed by Eglitis are used to follow the lineage of the resultant cells.

The Applicant's specification, teaches that stem cells comprise "only a small percentage of the total number of leukocytes in the bone marrow" (*See* application page 12, lines 7-8). As amended, claim 1 and dependent claims 2-4, recite the step of *"selecting mammalian stem cells* of myeloid origin capable of differentiating into neuronal cells." The application teaches selecting for stem cells of myeloid origin using markers, which are associated with human stem cells, such as CD34, Thy-1, KDR, class II HLA, etc (*See* application page 12, line 6 through page 14, line 2). In contrast, Eglitis does not teach or suggest a method of specifically *selecting for stem cells* of myeloid origin as disclosed by the application.

In summary, the Applicant's specification discloses methods of specifically *selecting for* the small percentage of the bone marrow cells that are stem cells, while Eglitis simply harvests and uses the unselected bone marrow. In light of the amendments, the Examiner is respectively requested to reconsider and withdraw the anticipation rejection.
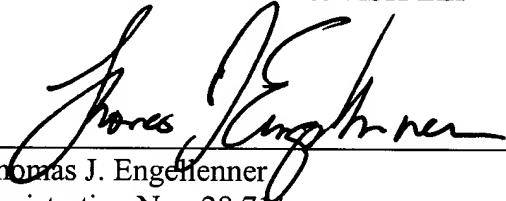
CONCLUSION

In summary, the above-identified patent application has been amended and reconsideration is respectfully requested for all the reasons set forth above. In the event that the amendments and remarks are not deemed to overcome the grounds for rejection, the Examiner is kindly requested to telephone the undersigned representative to discuss any remaining issues.

Respectfully submitted,

NUTTER McCLENNEN & FISH LLP

Date: ___September 3, 2003___

Thomas J. Engellenner
Registration No.: 28,711
Attorney for Applicants
World Trade Center West
155 Seaport Boulevard
Boston, MA 02210-2604
Tel: (617) 439-2948
Fax: (617) 310-9948

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neuronal markers in tissue culture (32); however, their ability to yield neuronal phenotypes in response to physiological signals in vivo has not previously been shown. Thus, our findings are not only of fundamental interest but also, once more robust, could have application as a cell-mediated therapy. Not only could neurons be contributed to the adult brain, but, if genetically engineered, they could be a potentially useful tool for treating disorders characterized by defective neuronal function or a loss of neurons such as Parkinson's disease, lysosomal storage disorders, psychiatric disorders, trauma, and other types of CNS injury.

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15. Isolated brains were minced with a razor blade, dissociated with proteases, washed, stained with TriChrome (TC)–conjugated rat antibody to mouse CD11b and allophycocyanin (APC)–conjugated rat antibodies to mouse CD45, and analyzed by flow cytometry.
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Turning Blood into Brain: Cells Bearing Neuronal Antigens Generated in Vivo from Bone Marrow

Éva Mezey,[1]* Karen J. Chandross,[2] Gyöngyi Harta,[1] Richard A. Maki,[3,4] Scott R. McKercher[3]

Bone marrow stem cells give rise to a variety of hematopoietic lineages and repopulate the blood throughout adult life. We show that, in a strain of mice incapable of developing cells of the myeloid and lymphoid lineages, transplanted adult bone marrow cells migrated into the brain and differentiated into cells that expressed neuron-specific antigens. These findings raise the possibility that bone marrow–derived cells may provide an alternative source of neurons in patients with neurodegenerative diseases or central nervous system injury.

Neural stem cells, the self-renewing precursors of neurons and glia, are the focus of intensive research aimed at developing transplantation strategies to promote neural recovery in the diseased or injured nervous system (1, 2). Recently, Bjornson *et al.* (3) demonstrated that neural stem cells could also differentiate into a variety of hematopoietic cells, including the myeloid and the lymphoid cell lineages, as well as more immature blood cells. Circulating T cells, B cells, and macrophages enter the brain (4–7). Rodent bone marrow cells migrate into the brain and differentiate into microglia and astrocytes when transplanted into previously irradiated recipients (8, 9). Recent evidence suggests that,

under experimental culture conditions, human and rodent bone marrow stromal cells can differentiate into cells bearing neuronal markers (10, 11). When transplanted into the lateral ventricle or striatum of mice, cultured marrow stromal cells migrate into the brain and differentiate into astrocytes (12, 13). There is evidence that other types of mesodermal-derived cells can also differentiate within the mammalian nervous system. For example, luteinizing hormone-releasing hormone (LHRH)–producing neurons originate from outside the central nervous system (CNS) and migrate into the hypothalamus (14). In the present study, we show that bone marrow–derived cells enter the brain and differentiate into cells that express neuronal markers, supporting the idea that mesodermal-derived cells can adopt neural cell fates.

Mice homozygous for a mutation in the *PU.1* gene were used as bone marrow transplant recipients. PU.1 is a member of the ETS (DNA binding domain) family of transcription factors and is expressed exclusively in cells of the hematopoietic lineage. In the

[1]Basic Neuroscience Program, [2]Laboratory of Developmental Neurogenetics, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA. [3]The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA. [4]Neurocrine Biosciences, 10555 Science Center Drive, San Diego, CA 92121, USA.

*To whom correspondence should be addressed. E-mail: mezey@codon.nih.gov

absence of donor bone marrow cells, PU.1 knockout mice lack macrophages, neutrophils, mast cells, osteoclasts, and B and T cells at birth (15, 16). These animals are born alive but require a bone marrow transplant within 48 hours after birth to survive and develop normally. There are no gross morphological differences in the brain cytoarchitecture of these mice versus wild-type mice. In the present study, PU.1 null mice were used as bone marrow recipients to optimize the number of cells derived from the donor and to permit an accurate estimation of the numbers of bone marrow cells that migrate into the nervous system.

NeuN, a nuclear protein that is found exclusively in neurons (17–19), was used as a neuronal marker. Specific NeuN immunoreactivity was not present in acutely isolated (20) bone marrow cells. Acutely isolated bone marrow cells were also examined for neural antigens in our transgenic mouse line in which oligodendrocytes and Schwann cells express LacZ (21). No LacZ-expressing or β-galactosidase–immunopositive cells were present, and there was no specific immuno-staining for NG2 chondroitin sulfate proteoglycan or O4, antigens that are present in Schwann cells and oligodendrocytes (22–24). These results strongly suggest that the bone marrow cell preparations were devoid of neurons and glia at the time of transplantation. When adult bone marrow cells were grown in culture for several weeks, the neural stem cell antigen, nestin, was present in 18% of the population [see Web fig. 1 (25)], indicating that bone marrow can give rise to neural stem cells.

Within 24 hours after birth, PU.1 homozygous recipients were given intraperitoneal injections of bone marrow cells from wild-type mice (20). Seven transplant recipient mice and nontransplanted control littermates were examined between 1 and 4 months of age. To determine the efficiency of the transplantation, we analyzed different organ tissues for the presence of donor-derived cells. Y chromosome–positive male cells were identified in hematopoietic organs of female recipients by fluorescent in situ hybridization histochemistry. Greater than 90% of spleen cells, in both white and red pulp, and ~10 to 15% of liver cells were Y chromosome–positive. All brains were examined by using a combination of in situ hybridization (ISH) to detect the Y chromosome and immunohistochemistry to visualize the neuronal nuclear marker, NeuN. Brains from a 4-month-old nontransplanted female [Fig. 1A and Web fig. 2, A to E (25)] and a nontransplanted male [Fig. 1B and Web fig. 2, F to J (25)] were processed together and served as controls for the Y chromosome hybridization specificity and efficiency (26). There was no specific Y chromosome staining in the female brain. The Y chromosome was frequently localized at the periphery of the nucleus, which is characteristic of heterochromatin (27, 28). The NeuN immunostaining was predominantly localized to the nucleus, although some neurons [as reported by others (19)] also exhibited perinuclear staining [Figs. 1 and 2 and Web figs. 2 to 5 (25)].

Marrow-derived cells (i.e., Y chromosome–positive) were present in the CNS of all of the transplanted mice examined. Between 2.3 and 4.6% of all cells (i.e., all identifiable nuclei, including vasculature) were Y chromosome–positive (Table 1). The

Fig. 1. Y chromosome staining in the CNS. Coronal sections from 4-month-old nontransplanted (**A**) female and (**B**) male brains were mounted and processed together. The panels show the overlay of the NeuN (red) immunostaining, Y chromosome nonradioactive ISH [visualized with tyramide-FITC conjugate (green)], and DAPI staining of cell nuclei (blue). The Y chromosome was restricted to the male brain, demonstrating hybridization specificity. (**C**) Confocal image of coronal sections from a 4-month-old recipient female striatum that was double-immunostained for the neuron-specific antigens NeuN and NSE. All NeuN-expressing cells (red) were also immunoreactive for NSE (green). (**D**) Sagittal section from a 1-month-old female PU.1 knockout mouse brain transplanted at birth with male bone marrow. The Y chromosome was visualized with BCIP/NBT (dark purple dots) to identify anatomical landmarks. cc, corpus callosum; cx, cerebral cortex; CPu, caudate putamen; fi, fimbria hippocampi; hi, hippocampus; LV, lateral ventricle. (**E** to **G**) Identical fields showing NeuN, Y chromosome, and DAPI nuclear triple staining in the hypothalamic dorsomedial nucleus of a 3-month-old female recipient. Colocalization of the Y chromosome [visualized with tyramide-FITC conjugate (green)] to a NeuN immunopositive (red) nucleus is shown in (E). In (F), DAPI staining identifies all cell nuclei (blue). Overlays of the NeuN, Y chromosome, and DAPI fluorescence are shown in (G). The arrow identifies a cell nucleus that contained both the Y chromosome (indicating the bone marrow origin) and NeuN. Scale bar in (G) represents the following sizes: 30 μm, (A) and (B); 10 μm, (C); 250 μm, (D); and 12 μm, (E) to (G). Similar results were observed with three different animals for each experimental condition.

Table 1. Quantitation of the number of donor cells in the forebrains of transplanted mice. A total of 21,682 cells was counted from seven animals. Ten to 20 random fields were photographed, and all DAPI-, NeuN-, or Y chromosome–positive nuclei were counted. Counts of cells represent an average from three independent investigators. The ratio of total cells to neurons was in good agreement with previous reports (45, 46).

Age (months)	DAPI-positive nuclei counted	NEU-positive nuclei counted	Y chromosome–positive cells	Y chromosome/NEU (double-labeled)	Neu-positive nuclei in all cells (%)	Y chromosomes in all cells (%)	Y of Neu-positive cells (%)
4	4831	1908	120	6	39	2.5	0.3
4	1322	221	60	5	17	4.5	2.3
3	3675	1483	130	16	40	3.5	1.1
3	4550	1825	105	15	40	2.3	0.8
2	3731	1039	162	16	28	4.3	1.5
1	1913	464	86	8	24	4.5	1.7
1	1660	380	76	7	23	4.6	1.8

Y chromosome–bearing cells were evenly distributed throughout the different brain regions [Fig. 1D and Web fig. 2, K and L (25)], in both white and gray matter. The Y chromosome was present in 0.3 to 2.3% of the NeuN-immunoreactive nuclei (Table 1). Confocal microscopy confirmed the presence of the Y chromosome in NeuN-immunopositive nuclei [Fig. 2 and Web figs. 4 and 5 (25)]. Y chromosome staining was localized to NeuN immunopositive cells and was not associated with any other neighboring nuclei in the x, y, or z planes. In the CNS of transplanted female mice, all NeuN-immunopositive nuclei were found in neuron-specific enolase (NSE)–containing cells (Fig. 1C). In the brain, NSE is expressed exclusively in neurons (29), demonstrating that Y chromosome–bearing cells can express two neuronal antigens. Most of these cells were found in the cerebral cortex [Web fig. 3, A to F (25)]; however, they were also present in the hypothalamus (Fig. 1, E to G), hippocampus, amygdala [Web fig. 3, G to I (25)], periaqueductal gray, and striatum. We did not detect Y chromosome–positive large motor neurons in the spinal cord or brainstem. A substantial number of Y chromosome–positive cell nuclei were present in cells within the choroid plexus of the lateral ventricle, in the ependyma of the ventricular system, and in the subarachnoid space, suggesting the cerebrospinal fluid as a primary route of entry [Web fig. 6 (25)]. We did not observe an overall increased density of Y chromosome–positive cell nuclei in neurogenic regions, including the subventricular zone, olfactory migratory region, or hippocampus. Because mesodermal stem cells can differentiate into microglia (8) and all microglia in these recipient animals arise from the donor bone marrow and are also Y chromosome–positive, we could not determine regional differences in the distribution of Y chromosome–positive nuclei.

These studies demonstrate that bone marrow cells migrate into the brain and differentiate into cells that express neuron-specific antigens. In combination with previous in vivo studies (9, 12, 13), the present work suggests that the bone marrow can supply the brain with an alternative 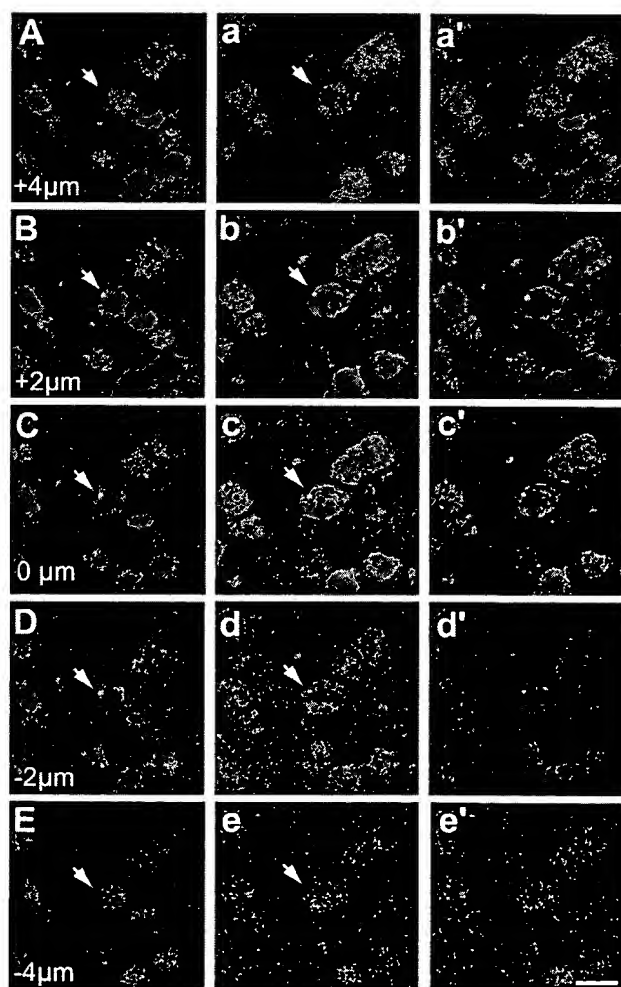source of neural cells. Neurons and macroglia (oligodendrocytes and astrocytes) are thought to arise from pluripotent neural stem cells that are present both in the developing (30) and adult mammalian CNS (31–35). It has been estimated that, for every 2000 existing neurons, one new neuron is produced each day (35, 36). In the rodent brain, there are two well-characterized neurogenic regions: one in the subgranular zone of the dentate gyrus and one in the forebrain subventricular zone (37–41). Two populations of neural stem cells have been identified in adult mammals: one in the ependymal cell layer lining the ventricles (33) and one in the subventricular zone [glial fibrillary acidic protein–immunoreactive cells (34), each of which gives rise to glial cells and neurons]. We suggest that, in addition to these sources of neural stem cells, there may be a continuous influx of bone marrow stem cells into the ependymal and subependymal zones that give rise to a variety of CNS neural cell types. An interesting possibility is that these entry routes might also serve as portals into the CNS for diseases that primarily originate in and affect the hematopoietic system (i.e., leukemia and AIDS).

Bone marrow is far more accessible than neural stem cells and has the added advantage of having inherent host compatibility, thereby obviating the need to screen for viral and foreign antigens. Although our study showed that only a small number of transplanted cells expressed neuronal antigens in the adult brain, there may be factors that promote the differentiation of bone marrow cells into distinct neural cell types. Once these factors are identified, bone marrow cells might be expanded in vitro and provide an unlimited source of cells for the treatment of CNS disease and injury. Because at least two different types of stem cells have been isolated from bone marrow (hematopoietic and stromal), characterizing the potential for each population will be an important step toward optimizing regenerative therapies.

Fig. 2. A NeuN- and Y chromosome–positive cell in the cingulate cortex (1.2 mm behind the bregma) of a 3-month-old homozygous female PU.1 knockout transplanted at birth with male bone marrow. The images were obtained with a Zeiss confocal microscope. (A to E) Five different levels through the section (1 μm thick each), overlaying the Y chromosome [visualized with tyramide-FITC (green) and DAPI (blue) staining]. (a to e) Overlays of the corresponding NeuN (red) and Y chromosome staining. (a' to e') Overlays of the corresponding NeuN, Y chromosome, and DAPI fluorescence. The Y chromosome hybridization was localized to a NeuN-immunopositive cell (arrow) and was not associated with any neighboring nuclei in the x, y, or z planes. Scale bar, 10 μm. These results were observed with five independent Z series from three different animals.

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20. PU.1 null mice were reconstituted as follows. Adult male mice (8 to 24 weeks old) were killed, and both femurs were removed under sterile conditions. The muscle was removed, and the ends of the bones were cut off with a scalpel. The remaining central portion of the femur was placed into Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (Gibco). Marrow cells from each femur were flushed out with medium. A suspension of the bone marrow cells was prepared by pushing the marrow and medium through 18-gauge, 21-gauge, and 25-gauge needles, consecutively. The cell suspension was centrifuged at 300g for 8 min, and the supernatant was discarded. The cells were washed in DMEM without serum, and an aliquot was removed for NeuN immunostaining. For the transplantation experiments, the remainder of live cells was centrifuged and resuspended in DMEM without serum. For immunostaining of acutely isolated bone marrow cells, see supplemental methods (25). Bone marrow transplants were performed as follows. At birth, each female neonate was given an intraperitoneal injection of a 0.05-ml suspension that contained 1 × 10⁷ male bone marrow cells (equivalent to one adult mouse). Approximately 0.05 to 0.5% of the total number of the marrow cellularity are hematopoietic stem cells and ~0.125% are stromal cells (42–44). All pups were given subcutaneous injections of enrofloxacin for 2 weeks, as previously reported (15), to help reduce the incidence of infection.
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26. Tissues were collected as follows. Reconstituted and normal mice were killed at the appropriate age with carbon dioxide gas. Tissues were collected and immediately stored at −80°C until used. ISH, histochemistry, and immunohistochemistry were performed as follows. Fresh frozen brain sections (12 μm thick) or acutely isolated bone marrow cells were fixed with 2 to 4% paraformaldehyde and immunostained with the neuronal nuclear marker NeuN [monoclonal immunoglobulin G1, 1:1000 dilution (Chemicon, Temecula, CA)]. The antibody was detected by using the Mouse on Mouse kit (Innogenex, San Ramon, CA) and subsequent deposition of biotinylated tyramide preceding the ISH. After the ISH, streptavidin-546 Alexa dye conjugate (Molecular Probes, Eugene, OR) was added to bind the biotin. Immediately following the deposition of the tyramide, nonradioactive ISH was performed on the same sections to detect the Y chromosome by using a 1.5-kb RNA probe, pY3531B, that was generated against a repeat sequence of the mouse Y chromosome (17) and labeled with digoxigenin–uridine 5'-triphosphate (for technical details, see http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html). After several washes, the digoxigenin was developed using an antibody to digoxigenin conjugated to either alkaline phosphatase (1:1500 dilution) or peroxidase (1:400 dilution) (Roche Pharmaceuticals, Indianapolis, IN). The antibody to digoxigenin was then visualized with either 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) as substrate (purple precipitate with light microscopy) or tyramide–fluorescein isothiocyanate (FITC) (NEN, Boston, MA) (green fluorescence). Subsequently, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue fluorescence). Representative sections from transplanted mice were double-labeled with NeuN and NSE [polyclonal, 1:10,000 dilution (Polysciences, Warrington, PA)] antibodies. Primary antibodies were visualized

with an Alexa 594 antibody to mouse (NeuN, 1:1000 dilution, Molecular Probes) or Alexa 488 secondary antibodies to rabbit (NSE, 1:500 dilution, Molecular Probes).
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47. E.M. dedicates this report to the memory of János Szentágothai (1912–94), anatomist, statesman, romantic, artist, and mentor, who helped me understand the difference between looking at tissue sections and seeing the secrets they hold. The authors would like to express their sincere thanks to R. Dreyfus for his help with the conventional microscopy and C. L. Smith and R. Cohen for their help with the confocal microscopy. We are also grateful to M. Brownstein, R. Cohen, H. Gainer, L. Hudson, and M. Palkovits for their helpful suggestions and support throughout the work. These studies were supported by NIH grant AI30656 to R.A.M.

Coding the Location of the Arm by Sight

Michael S. A. Graziano,* Dylan F. Cooke, Charlotte S. R. Taylor

Area 5 in the parietal lobe of the primate brain is thought to be involved in monitoring the posture and movement of the body. In this study, neurons in monkey area 5 were found to encode the position of the monkey's arm while it was covered from view. The same neurons also responded to the position of a visible, realistic false arm. The neurons were not sensitive to the sight of unrealistic substitutes for the arm and were able to distinguish a right from a left arm. These neurons appear to combine visual and somatosensory signals in order to monitor the configuration of the limbs. They could form the basis of the complex body schema that we constantly use to adjust posture and guide movement.

Without an accurate sense of the position of the limbs, head, and torso, we would be unable to guide movement, process the spatial location of nearby objects, or distinguish our own body parts from external objects. People with damage to their parietal lobes can have difficulty in all of these dimensions (1, 2). Studies in normal humans show that the body schema is not simply a representation of joint angles, but a complex integration of vision, proprioception, touch, and motor feedback (3–6). Although a great deal is known about the processing of joint angle and muscle stretch in the somatosensory system (7), little is known about how different sensory modalities are combined by neurons in the parietal lobe or elsewhere to construct the body schema (8, 9).

The present set of studies focused on the coding of static arm position. The sense of

arm position depends on many sources of information, including proprioception and vision (3–6, 10–12). Here we show that neurons in parietal area 5 of the monkey brain, but not in the primary somatosensory cortex, respond in relation to the seen position of a false arm. They are also sensitive to somatosensory signals, responding in relation to the felt position of the monkey's actual arm. These somatosensory and visual signals are combined in individual neurons to provide a possible code for static limb position.

Responses of single neurons in area 5 were studied in two monkeys (13). The recording site in monkey 1 is shown in Fig. 1A, and the apparatus is shown in Fig. 1B. The arm contralateral to the recording electrode was outstretched, and the ipsilateral arm was held close to the body (not shown). The arms were covered with a black plastic plate. On top of the plate, a realistic false arm was placed in the monkey's view. This false arm was from a monkey of the same species and had been prepared by a taxidermist. The cut end was covered from view by a portion of

Department of Psychology, Princeton University, Princeton, NJ 08544, USA.

*To whom correspondence should be addressed. E-mail: graziano@princeton.edu

Transplanted bone marrow generates new neurons in human brains

Éva Mezey*†, Sharon Key*, Georgia Vogelsang‡, Ildiko Szalayova§, G. David Lange¶, and Barbara Crain‡

*National Institutes of Health (NIH)/National Institute of Neurological Disorders and Stroke (NINDS)/In situ Hybridization Facility (ISHF) and §NIH/National Institute of Mental Health/Laboratory of Genetics (LOG), Building 36, 3D06, Bethesda, MD 20892; ‡Johns Hopkins University, School of Medicine, Baltimore, MD 21287; and ¶NIH/NINDS/Instrument and Computer Section, Building 36, 2A03, Bethesda, MD 20892

Adult bone marrow stem cells seem to differentiate into muscle, skin, liver, lung, and neuronal cells in rodents and have been shown to regenerate myocardium, hepatocytes, and skin and gastrointestinal epithelium in humans. Because we have demonstrated previously that transplanted bone marrow cells can enter the brain of mice and differentiate into neurons there, we decided to examine postmortem brain samples from females who had received bone marrow transplants from male donors. The underlying diseases of the patients were lymphocytic leukemia and genetic deficiency of the immune system, and they survived between 1 and 9 months after transplant. We used a combination of immunocytochemistry (utilizing neuron-specific antibodies) and fluorescent in situ hybridization histochemistry to search for Y chromosome-positive cells. In all four patients studied we found cells containing Y chromosomes in several brain regions. Most of them were nonneuronal (endothelial cells and cells in the white matter), but neurons were certainly labeled, especially in the hippocampus and cerebral cortex. The youngest patient (2 years old), who also lived the longest time after transplantation, had the greatest number of donor-derived neurons (7 in 10,000). The distribution of the labeled cells was not homogeneous. There were clusters of Y-positive cells, suggesting that single progenitor cells underwent clonal expansion and differentiation. We conclude that adult human bone marrow cells can enter the brain and generate neurons just as rodent cells do. Perhaps this phenomenon could be exploited to prevent the development or progression of neurodegenerative diseases or to repair tissue damaged by infarction or trauma.

N eurogenesis used to be thought to be completed during embryonic life in rodents as well as humans. During the last decade, however, numerous studies have suggested that neurogenesis continues in adult animals and humans, at least to a certain extent in a few privileged areas of the brain (1–4). Most of these studies have focused on endogenous neural progenitor cells (neural stem cells) localized in the subventricular zone of the lateral ventricle and in the dentate gyrus in the hippocampus in rodents (4). In the monkeys these cells are present in the hippocampus and neocortex (5, 6). Likewise, Eriksson *et al.* (7) found that new neurons are generated continuously in the human dentate gyrus throughout life.

It is also conceivable that stem cells from other sources might enter the brain and form neurons there. Uchida *et al.* (8) isolated CNS stem cells from human fetal tissue and transplanted them into the brains of mice, where they subsequently proliferated and differentiated into neuronal cells. One source of such cells in the brain could be the bone marrow. Adult bone marrow stem cells seem able to differentiate into muscle, skin, liver, lung, and neural cells in rodents (9–18). Furthermore, transplanted bone marrow cells in humans have also been shown to form myocardial cells (19, 20), hepatocytes (21, 22), and epithelium of the skin and gastrointestinal tract (20). Because we have demonstrated previously that transplanted bone marrow cells migrate into the brains of mice and give rise to neurons there (15), we hypothesized that the same thing might occur in the human CNS after bone marrow transplantation. We tested this hypothesis by

looking for Y chromosome-positive neuron-like cells in postmortem brain samples from females who had received bone marrow transplants from male donors.

Methods

Four female patients who had had bone marrow transplants from male donors were selected from the autopsy files of The Johns Hopkins Hospital. Patient 1 had Omenn's syndrome, was transplanted at 9 months of age, and died 10 months later. Patient 2 had Hodgkin's disease and was transplanted at 34 years of age. Patient 3 had acute lymphocytic leukemia and was transplanted at 10 years of age. Patient 4 had acute lymphocytic leukemia and was transplanted at 20 years of age. Patients 2–4 died within ≈2 months of receiving their transplants.

Formalin-fixed, paraffin-embedded sections (6 μm) from the following brain areas were examined in each case: neocortex, striatum including the lateral ventricular wall, hippocampus with adjacent mesial temporal lobe structures, and cerebellum. Sections from three nontransplanted female patients were used as negative controls for Y-chromosomal staining. Sections from four male patients were used as positive controls. One tissue sample each from a male and a control female brain were reembedded together into one paraffin block and serially sectioned at a thickness of 6 μm; sections from this block were used as controls in all experimental series.

After deparaffinization in Citrisolv (Fisher Scientific) the sections were rehydrated, and heat-induced antigen retrieval was performed in a histology microwave oven by using a citrate buffer (Citra-plus, Innogenex, San Ramon, CA) for 5 min at 600 W. Next, immunostaining was performed by using primary antibodies that were detected by the Sternberger peroxidase antiperoxidase (PAP) method (23) followed by either biotinylated tyramide (for Kv2.1) or FITC-tyramide plus [(for neuronal nuclear antigen (NeuN)] (Perkin–Elmer). The primary antibodies used were directed against two neuronal proteins: NeuN (24), a neuron-specific nuclear protein, and Kv2.1, a neuron-specific voltage-gated potassium channel antibody (25, 26). Kv2.1 was recognized as a neuron-specific potassium channel that was first described in the principal neurons of the hippocampus and cortex and later shown to be present in the vast majority of interneurons as well (26). The NeuN antibody was a mouse monoclonal (used at 1:1,000) (Chemicon), and the Kv2.1 antibody was a rabbit polyclonal (used at 1:500, Alomone, Jerusalem). After immunostaining, the *in situ* hybridization was carried out as described (15) by using a digoxigenin-labeled riboprobe complementary to the satellite region of the human Y chromosome. We prepared the template from which we made probe from human genomic DNA by using primers that amplified a 1.3-kb-long DNA of the human Y chromosome. The probe was visualized by using peroxidase-conjugated antidigoxigenin antibody (Roche, Indianapolis) followed by a tyramide-CY3 fluo-

rochrome plus (Perkin–Elmer) amplification. At the end, all sections were stained with a 1% Sudan-black solution to mask lipofuscin-induced autofluorescence, which is a common problem when working with human brain tissue (27). All sections were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma), a chromosomal stain to label nuclei, and mounted with 80% glycerol/20% Tris. A separate series of sections was hybridized by using a radiolabeled Y chromosome probe, which is easily visualized at low magnification. This enabled us to count Y-positive cells and examine their overall distribution. All sections were viewed with conventional Leitz and confocal Zeiss microscopes.

In addition to the nontransplanted male and female brains, to make sure of the specificity of the techniques we ran as controls immunostaining with amplification without primary and/or without the secondary antibodies.

Analysis of the distribution of Y chromosome-positive cells was done as follows. The Y chromosome probe was radiolabeled with [^{35}S]UTP and hybridized to brain sections. After emulsion coating and autoradiographic development of the sections, the Y-positive nuclei were counted at low magnification ($\times$10). The counts in individual 1.2-mm^2 microsquares (visual field) of a grid that covered the entire section were determined. The mean and

variance of the counts and the variance-to-mean ratio were calculated. In a random (Poisson) distribution, the variance-to-mean ratio is 1. Therefore, the nearness to 1 of this ratio is a measure of the randomness of the distribution (28).

Results

In the control male brain sections we detected the Y chromosome with both the fluorescent and autoradiographic techniques in >90% of the nuclei, whereas no labeling was observed in the brain sections of female patients who have not received transplants. In each of the transplanted patients examined we readily detected Y-positive cells by means of autoradiography. In all patients using conventional fluorescent microscopy we observed double-labeled cells that were positive for both the Y chromosome and one of the neuronal markers as shown in an example in Fig. 1. Most of the cells that were double-labeled with the Y chromosome and the neuronal markers were detected in the hippocampus and the neocortex of patients (Fig. 2). The antibody that binds to Kv2.1 gave a very convincing staining of neuronal somata and dendrites in all cortical areas and in the hippocampus (as described in the literature; see ref. 26), and also a few axons seemed to show a patchy immunostaining after signal amplification.
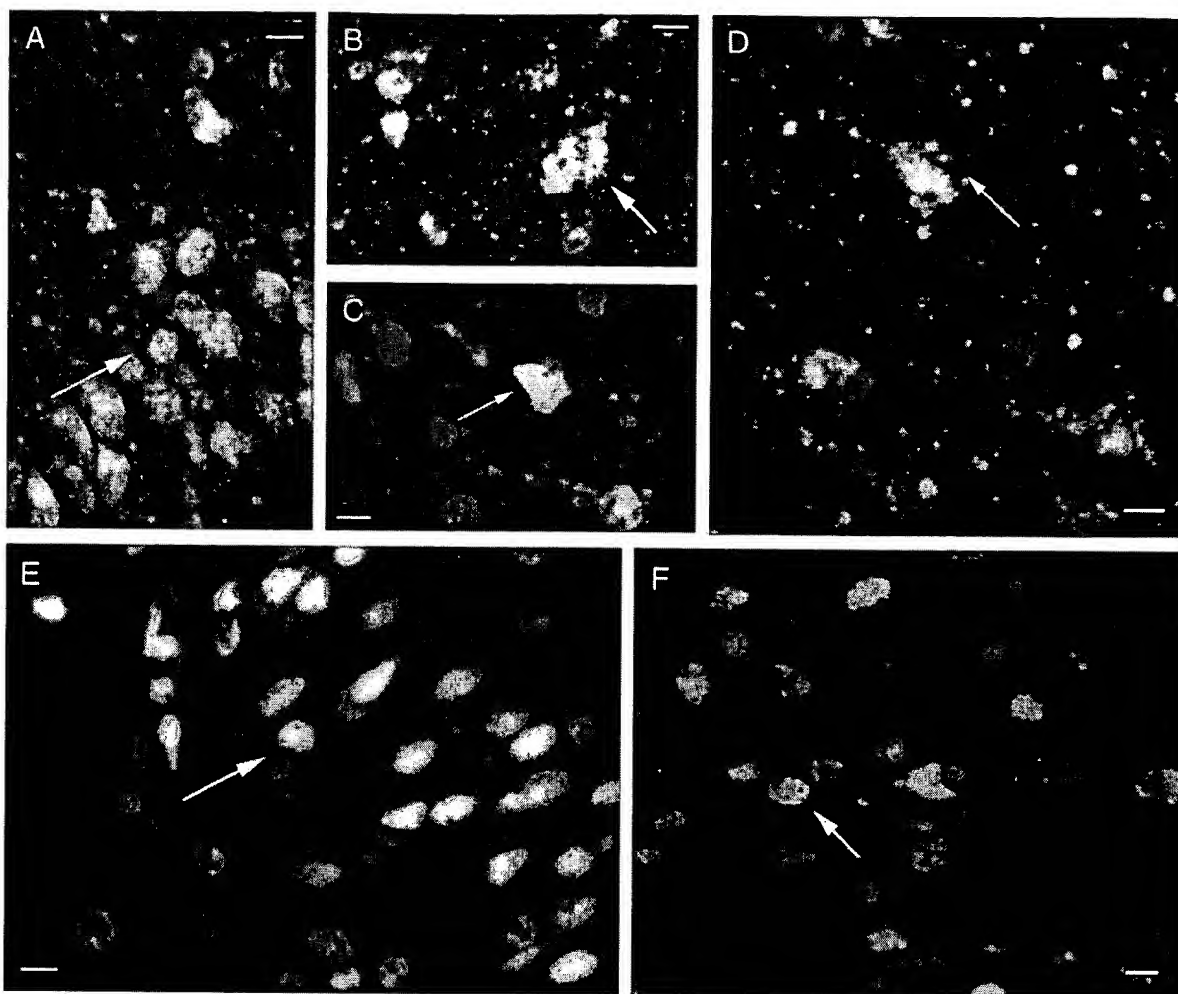
Fig. 2. Neuronal markers colocalized with the Y chromosome. Fluorescent microscopic images of neocortex from patients 2 (*A–C*) and 1 (*E*) and hippocampus from patients 1 (*D*) and 3 (*F*) are shown. The green color represents the immunostaining for neuronal markers Kv2.1 (*A–D*) and NeuN (*E* and *F*), and the Y chromosome is represented by the red fluorescent dots. All cell nuclei are stained with 4′,6-diamidino-2-phenylindole, a chromosomal marker that shows up as blue fluorescence. All images are overlays of the images seen through the three separate filters to show all colors. Arrows point to cells that are labeled with neuronal markers and are also Y chromosome-positive. In the Kv2.1 immunostaining the initial axons of some neurons can also be visualized. (Scale bars, 10 μm.)

Confocal z series confirmed the presence of Y chromosomes in the nuclei of the same cells that were immunopositive for the neuronal markers. Examples from two patients are shown in Fig. 3. In the patient with the greatest number of double-labeled cells (patient 1), the distribution of the Y-positive cells suggested that clonal expansion had occurred: We detected no labeled cells in many visual fields but typically saw clusters of positive cells when such cells were detected. This pattern was easier to recognize in the autoradiographic sections, which could be examined at low magnification. In several clusters, the Y-positive cells were both neuronal and nonneuronal. In the cortex, based on morphology and location, the Y-positive neurons were small pyramidal cells, whereas in the hippocampus the Y-positive cells seemed to be granule cells. We also saw Y-positive cells in the white matter, some of which looked like oligodendrocytes based on the shape, size, and arrangement of their nuclei. We saw many Y-positive cells inside vessels (in cross section) and also some Y-positive endothelial cells in the vascular wall.

We counted all the Y chromosome-positive cells in two entire sections of striatum/cortex and hippocampus/cortex blocks from two patients, analyzing 281 and 247 microscopic fields from patients 1 and 3, respectively (Table 1). In patient 1 we found 519 Y-positive cell nuclei among 182,000 nuclei (stained with

ethidium bromide), and in patient 3 we found 1,842 Y-positive nuclei among 196,700. In the same sections we found 19 and 5 Y chromosome-containing nuclei, respectively, that also colocalized with neuronal antigens. Based on these observations and conservatively assuming that 25% of all nuclei in the human brain are neurons [this number in the cortex of primates varies between 27% and 60% (29, 30)], one in every 2,000–4,000 neurons might derive from the bone marrow.

When we evaluated the distribution of Y chromosome-positive cells in patients 1 and 3 we found that it was not random. The variance-to-mean ratios of all counts in microsquares overlaying the sections studied were calculated to be 10.2 (patient 1) and 4.7 (patient 3).

Discussion

Several studies have shown that there is neurogenesis in the adult brain, even though it may be limited. Cells with neuron-specific markers can be formed by neural stem cells *in vivo* (1, 3, 31) as well as by bone marrow cells *in vitro* (32, 33) and *in vivo* (9, 15). Recently, Priller *et al.* (34) published a picture of a well differentiated, enhanced GFP-positive Purkinje cell in the cerebellum of a mouse transplanted with enhanced GFP-tagged bone marrow from a second animal. Along with earlier studies, this finding
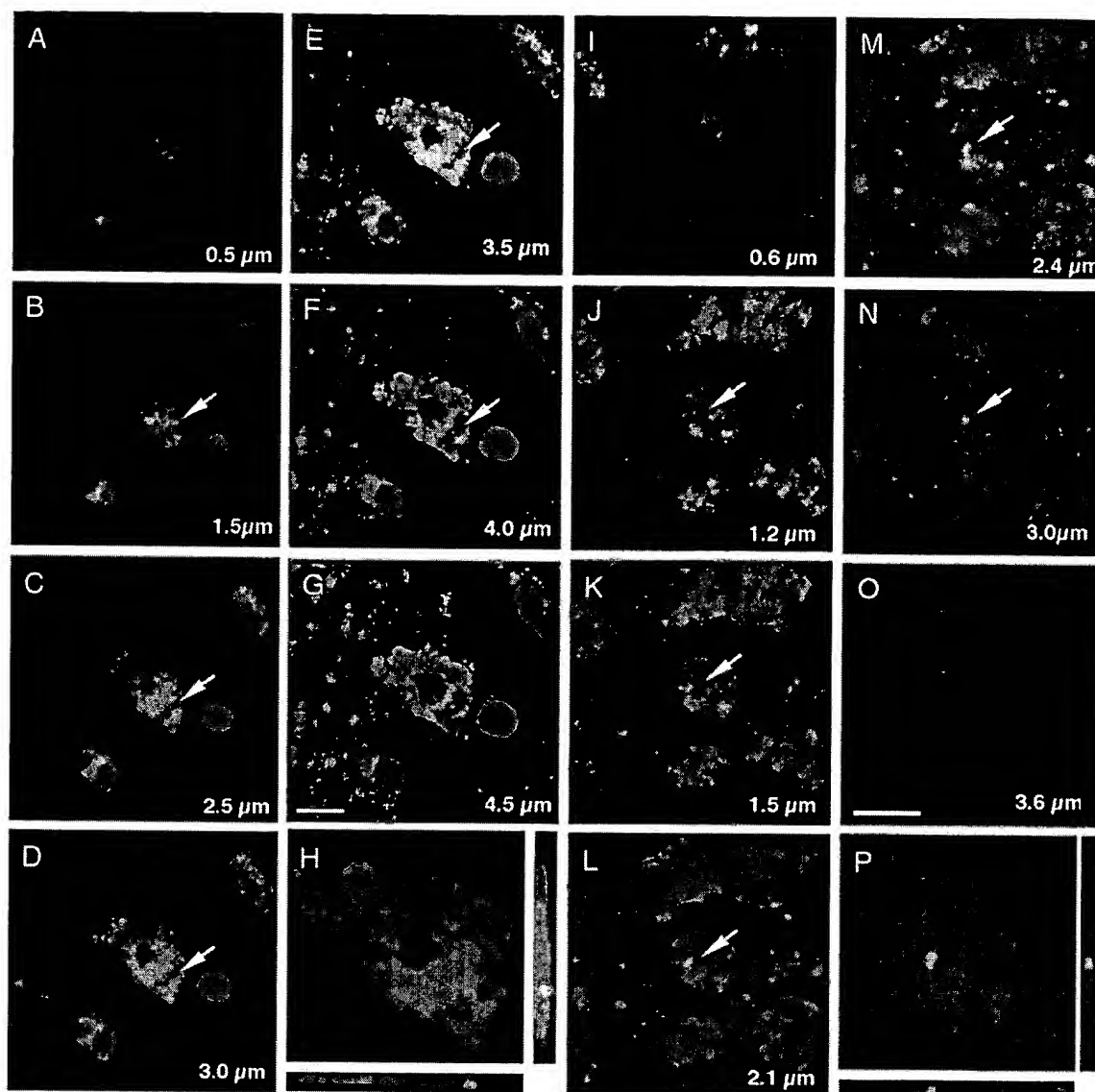
Fig. 3. Two confocal z series are shown. (*A–D*) Optical sections (1-μm-thin) of a neocortical neuron from patient 2. (*E–F*) Optical 1-μm-thin slices of a hippocampal granule cell from patient 1. Both cells are immunostained with the neuronal marker Kv2.1 (green); the Y chromosome is red (CY3-plus), and the nucleus is blue (4′,6-diamidino-2-phenylindole). The arrowheads point to the double-labeled cells. Note that the cell nucleus and the Y chromosome are consistently in the same plane.

was a significant step toward showing that fully functional neurons can be generated from bone marrow cells. Based on our present study, we have no way of knowing which population of bone marrow cells entered the CNS and differentiated into cells expressing neuronal morphology and neuronal markers. Mesenchymal stem cells have been shown to differentiate into many different cell lineages (35). All of our patients received total bone marrow transplants containing both hematopoietic and mesenchymal stem cells.

Recently, two studies (36, 37) suggested the possibility that Y-positive cells of bone marrow origin might simply fuse with embryonic stem cells instead of transdifferentiating into cells characteristic of various tissues. Although this idea is interesting, no data support its relevance *in vivo* (see ref. 38). In fact, the fusion process is very inefficient *in vitro*: 1 in a million, which is a figure much lower than the number of double-labeled cells that we see in the brain. Recently, Castro *et al.* (39) failed to detect blue cells in the brains of lethally

Table 1. Number of Y chromosome-positive (Y⁺) cells in two patient samples

Patient	No. of visual fields (1 field = 0.94 mm²)	No. of Y⁺ nuclei	No. of all nuclei	No. of Y⁺ nuclei in cells with neuronal markers
1	281	519	182,000	19
3	247	1,842	196,700	5

irradiated mice that received bone marrow from Rosa26 mice with a β-galactosidase-containing transgene. The use of protein products of transgenes as markers to follow graft fate is plagued with problems, but to date these may not have been emphasized enough. It is practically impossible to achieve ubiquitous transgene expression. Transgenes, including those driven by the Rosa26 promoter, suffer from instability in several tissues. Furthermore, to detect low levels of lacZ is difficult, and the detection is sensitive to fixation and staining conditions (40). Because one would have expected to see at least labeled microglia in the brain, one must assume that the results reported were due to technical problems.

In the present cases it is difficult to make general statements about the extent of CNS colonization or to conclude whether cells enter the brain continuously or just at the time of the transplant. All of our patients received irradiation to eliminate the underlying disease. We do not know how much effect the irradiation might have on the migration of circulating cells into the brain by either injuring the blood–brain barrier and/or releasing possible recruiting factors due to the injury caused by the radiation. It is known, however, that circulating blood cells are able to enter the brain parenchyma freely in healthy subjects (41). We studied a limited number of patients with different diseases, ages, survival times after transplant, and treatments of postmortem tissue. Because of the technical difficulties of double-labeling cells in paraffin-embedded postmortem tissue and the fact that we had to reduce the sensitivity of the method to eliminate background, we believe that our numbers are in fact lower that the real number of differentiating cells *in vivo*. Although we found donor (i.e., Y chromosome-positive) cells exhibiting two specific neuronal markers (NeuN and Kv2.1) in the hippocampus and neocortex in all three of the patients with technically satisfactory samples, the numbers of double-labeled cells were much lower than those reported in rodents (9, 15). We found 2–5 Y-positive neurons per 10,000 human neurons vs. 50 per 10,000 rodent neurons. Whether this is a species difference is unclear. The sections with the highest number of newly formed neurons (7 per 10,000

neurons) were from patient 1, the youngest studied, who had her transplant in infancy and also had the longest posttransplant survival time. All the other patients lived only weeks after the transplant, and there was no significant difference in the number of donor-positive cells in their brains.

It should also be noted that among the Y-positive cells, neurons were consistently in the minority. Based on their nuclear morphology, size, and location, we feel that the nonneuronal cells bearing the Y chromosome were a mixture of oligodendrocytes, astrocytes, and possibly microglia. We also detected endothelial, meningeal, and ependymal cells that were Y-positive. Finally, many circulating white blood cells within the vascular lumen were also Y-positive, as one would expect.

Our analysis showed that the distribution of Y-positive cells within the brain is not random. Instead, the cells appeared in clusters, a spatial distribution that suggests nondifferentiated cells may enter an area and then further propagate there. In these areas we see different kinds of Y-positive cells (neuronal and nonneuronal). One possibility is that one undifferentiated cell migrates into an "area of need" and then goes through asymmetrical divisions to produce different lineages of cells. Another possibility is that many progenitor cells are "called in," and they differentiate into different lineages of cells. Whichever is the case, we speculate that areas in need of new cells (because of physiological turnover or pathological loss of cells) may be able to signal to potential stem cells to coax them into the region, and then clonal expansion occurs to help restore the number of cells to normal. Therefore, it will be very important to try to find the factors responsible for inducing stem cells to migrate into lesioned or sick areas of the brain. Discovering these factors could aid the attempt to use bone marrow cells to repair the brain.

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